TORREYA

May, 1911

Vol. 11 No. 5

THE NATURE AND FUNCTION OF THE PLANT OXIDASES

By Ernest D. Clark

(*Continued from April Torreya*)

Pathology

In most of the cases first considered, the oxidases played a beneficial or useful part in the activities of plant life, but we are now to see that under certain conditions they may cause pathological processes. There is a disease of tobacco known as the "mosaic disease" which is characterized by the checkered appearance of the green leaves, these checkered places being yellow. In 1902, Woods[55] showed that rapid growth caused by cutting back often induced this disease, which he attributed to the abnormal activity of the oxidases. He believed the trouble was caused by an excessive activity of these enzymes due to lack of nitrogenous and other foods in the cells, which if present in normal quantities, seem to enable the cells to keep the oxidases within bounds. The diseased portions of the leaves showed the presence of great quantities of oxidases, but exhibited a striking lack of starch, nitrogenous matter, etc. In the so-called "mulberry dwarf" disease of the mulberry tree in Japan, Suzuki[56] found the same state of affairs. When the mulberry trees were repeatedly cut back, they developed a wrinkled and yellow appearance of the leaves, accompanied by a great increase of oxidases in the yellow portions, and also by a lack of plant foods in the diseased places. Suzuki thought that anything inter-

[55] Woods. Observations on the Mosaic Disease of Tobacco. Bull. 18, Bur. Plant Industry, U. S. Dept. Agric. 1902.

[56] Suzuki. Mulberry Dwarf Troubles in Japan. Bull. Agric. Coll. Tokyo, 4: 167 and 267. 1900.

[No. 4, Vol. 11, of Torreya, comprising pp. 77–100, was issued 19 April 1911.]

fering with the proper translocation of foods to rapidly growing parts would permit an abnormal development of oxidases and a consequent yellow or diseased condition. Woods[57] discovered that oxidases, when acting in the sunlight, have the power to destroy chlorophyll and cause yellow spots on leaves; a condition noted on the foliage of the Bermuda lily, carnation, tomato, etc. Punctures of leaves by insects or the presence of parasitic fungi, most of which contain oxidases, result in the decomposition of chlorophyll and the production of such yellow spots. Oxidases may exist in the soil or plant remains for several months, and thus cause infection if the new plants are not in a healthy condition. Recently Hasselbring and Alsberg[58] found that there is a disease of cabbage and spinach somewhat like the " mosaic disease " of tobacco. They also noted an apparent increase of oxidase content in the diseased spots, but thought this result might be caused by a decrease of anti-oxidases in the affected area.

Experimental Part

The historical part of this paper makes it evident that there has been no lack of effort to determine the distribution and nature of the oxidizing enzymes. However, many previous investigations were carried out with the use of but one reagent, which was generally guaiac tincture; besides, adequate checks upon the reagents or upon the plant juices were not made. Any one familiar with the use of the oxidase reagents realizes that the most sensitive of them, such as the indo-phenol reagent and phenolphthalin, are so easily oxidized that constant care must be taken that the action of atmospheric oxygen be not interpreted as a positive test for a weak oxidase. Furthermore, in all investigations involving the use or comparison of colors, one must be alert to detect differences due to a personal factor or to the illumination. Our investigation was undertaken with the purpose of examining and extending previous work upon the distribution of the oxidases; studying the conditions of their activity, and their effects upon different reagents, etc.

[57] Woods. The Destruction of Chlorophyll by the Oxidizing Enzymes. Centralblt. f. Bakt. II Abt. 5: 745. 1899.

[58] Hasselbring and Alsberg, *loc. cit.*

The Nature of the Investigation

The object of our experiments may be formally stated as follows:

(*a*) To study the distribution of the oxidases and of catalase in the higher plants, beginning with the lowest; using representatives of as many available orders and families as possible. To make the data more systematic and to reveal, if possible, any natural relationships, the results are tabulated according to the botanical classification.[59]

(*b*) To examine as many plant parts as possible, to see if there is a localization of the oxidases in special organs.

(*c*) To use a series of different oxidase reagents upon each sample, and to repeat all tests, under parallel conditions, with boiled controls in every case. Our purpose in this was to detect any differences in the behavior of the several reagents when used under controlled conditions upon a large number of materials of plant origin.

(*d*) To determine the extent of the distribution of those chromogens in plants which are oxidized to colored compounds by the natural oxidase of the plant itself. These chromogens are the so-called "respiration pigments" of Palladin.

The Methods of the Investigation

The method of preparing the enzyme solution varied with the nature of the material. Fleshy parts that were sufficiently large were run through a meat-chopper, smaller ones were grated on a vegetable grater, while leaves, flowers, etc., were macerated in a mortar. Control experiments proved that the iron of the grater had no effect. In whatever manner the material was finely divided, it was then treated with distilled water and allowed to stand for fifteen minutes. The volume of distilled water varied with the amount and nature of the material. After standing for fifteen minutes with distilled water, the extract

[59] For full details of experimental work and for the arrangement of results according to the botanical classification see the original dissertation upon which this paper is based.

thus obtained was filtered through muslin.[60] These clear solutions were made up to 50, 100 or 200 cubic-centimeters, depending upon the amount of material used in the preparation of the extracts.

The tests were carried out in the following manner: 5 c.c. of the plant extract were placed in each of a series of test-tubes and to each such portion of extract ten drops of reagent were added from a dropping bottle. This was a test for the *oxygenases* (direct oxidases) and was repeated in every detail, except for the addition of five drops of 1 per cent. pure hydrogen peroxide solution,[61] when testing for peroxidase. The latter treatment caused an increase of coloration, when compared with the corresponding oxygenase effects, if *peroxidase* were present. Boiled portions of the enzyme solutions were tested in precisely the same manner for control purposes. Portions of the extracts were tested again after standing one hour, and once more after the lapse of twenty-four hours, to reveal any subsequent change in the action of the oxidases. The presence of *catalase* was shown by the evolution of gas when five drops of 1 per cent. hydrogen peroxide solution were added. Any change of color indicating chromogens or any peculiar appearance of the plant juices were noted.

It became evident very early in our work that failure to obtain a positive test for oxidases usually indicated the presence of acids; so we determined the acidity of many of the extracts by titrating ten cubic-centimeter portions with $N/10$ potassium hydroxide solution, using phenolphthalein as the indicator. To serve as a further check on our results, all of these tests were made on *another* day with *another* sample of the material to obviate the effects of any psychological differences on the observer's part, or individual variations in the plants examined.

[60] This muslin had previously been treated with boiling dilute hydrochloric acid solution. It was then washed with water, treated with boiling dilute ammonium hydroxid solution, washed with distilled water until neutral, and finally dried in a dust-free place.

[61] The best hydrogen peroxide is the "Perhydrol" of Merck, containing 30 per cent. of H_2O_2. It was diluted with twenty-nine volumes of water. This product is practically neutral and contains no preservative.

Naturally, the collection and recording of all these data pertaining to over a hundred separate plants and plant parts was no mean task, and to facilitate the process as much as possible we had mimeographed sheets prepared with appropriate columns so that the labor of recording and preserving many hundreds of observations was reduced to a minimum.

As reagents for the oxidases, we used ordinary *guaiac tincture*, also tincture of guaiacum which had been boiled with bone-black to remove peroxides,[62] α-naphthol, the hydrochloride of para-phenylene-diamine, phenolphthalin, the indo-phenol reagent and phenol. Both the ordinary and purified guaiac tinctures were 2 per cent. solutions of gum guaiacum in absolute alcohol. These tinctures give a blue color when oxidized.

The *α-naphthol reagent* had a concentration of 1 per cent. of the substance in a 50 per cent. aqueous solution of alcohol. It gives a lavender color when oxidized.

The *para-phenylene-diamine* solution contained 1 per cent. of the hydrochloride in distilled water. This reagent yields a greenish color when oxidized.

The *phenolphthalin reagent* was made according to Kastle's method.[63] We treated a pinch of phenolphthalin with 1 c.c. of N/10 NaOH solution, dissolved as much of it as possible, then added 25 c.c. of water, filtered and made up to 100 c.c. We used 5 c.c. of this solution plus 10 c.c. of the extract to be tested for the oxidase, let the mixture stand fifteen minutes, then made it alkaline with N/20 NaOH solution, when the mixture, in the presence of oxidases, acquired a pink or red color due to the phenolphthalein resulting from the oxidation of the colorless phenolphthalin.

The *indo-phenol reagent* was applied by adding two or three drops of a 1 per cent. solution of α-naphthol in 50 per cent. alcohol and an equal amount of a 1 per cent. aqueous solution of para-phenylene-diamine hydrochloride to the extract to be tested, then making the mixture slightly alkaline with sodium

[62] Moore and Whitley. The Properties and Classification of the Oxidizing Enzymes, etc. Biochem. Jour. 4: 136. 1909.

[63] Kastle, Chemical Tests for Blood. Bull. 51, Hyg. Lab'y, U. S. Pub. Health and Marine Hospital Service, Washington, 1909, p. 25 ff.

carbonate solution, which caused the purple oxidation product to dissolve.

Phenol was used in a 5 per cent. aqueous solution and became reddish brown in twenty-four hours if oxidized.

The phenolphthalin and indo-phenol reagents oxidize spontaneously in the air and must be freshly prepared for satisfactory use.

In testing for the chromogens in the various plants we merely allowed some of the juice to stand for twenty-four hours, when the chromogens became evident by being changed by the oxidases to the colored state, generally brown, reddish or black.

For the detection of oxidases in plant sections, under the microscope, one may use the α-naphthol reagent described above, either with or without hydrogen peroxide. Under these conditions oxidizing tissues or cells soon stain violet or lavender and make a beautiful picture until the diffusion of the oxidases is complete and the whole preparation becomes dark. Sections of vines containing much food-conducting tissue, such as *Aristolochia macrophylla*, stain very strikingly as a result of this treatment.

Summary of Oxidase Tests

Specimens Examined	Oxygenase	Peroxidase	Catalase	Chromogens
All parts (110)	**55**	**78**	**105**	**30**
Leaves (17)	12	12	16	6
Floral organs (20)	8	11	20	7
Tubers, bulbs, etc. (21)	14	20	19	7
Fruit (41)	13	28	40	7
Other parts (11)	8	7	10	3

Study of the Effect of Acidity upon Oxidases

In the course of our systematic search for the oxidases, it soon became evident that an acidity in the plant juices and extracts greater, per 10 c.c. of plant liquid, than the alkalinity of 0.8 c.c. of N/10 KOH solution, with phenolphthalein as the indicator, usually indicated the absence of oxidases in the plant part under examination. These observations led the writer to study this phenomenon further. It was found that 10 c.c. of lemon juice required 18.5 c.c. of N/10 KOH solution for neutralization, and did not show the presence of oxidases either before or after neutraliza-

tion. Three or four drops of a coffee-bean extract showing a very high oxidase activity were added to 10 c.c. of fresh lemon juice, with the result that the oxidase action was inhibited, but immediately after neutralization the oxidase caused a faintly positive test. This same experiment was repeated, using 9.25 c.c. of N/5 acetic acid solution, the N/5 solution being used to make the total acidity equal to that of the lemon juice and to keep the total volume always the same (10 c.c.), with the addition of distilled water and a few drops of coffee-bean extract as before. To our surprise this apparently did not affect the oxidase at all, for a very strong coloration was obtained with guaiac tincture, etc. Then the experiment was repeated in exactly the same manner upon mixtures containing 9.25 c.c. of N/5 H_2SO_4, HCl, and citric acid solutions. The results were the same in the three cases: the oxidase reaction was completely inhibited and after neutralization with calcium carbonate or potassium hydroxid, a faint bluish coloration of guaiacum was detected in the citric acid test-tube. The rest were negative after neutralization. The sulphuric acid mixture was neutralized with calcium carbonate and divided into two portions, to one of which fresh coffee extract was added, to the other some fresh guaiac tincture; no bluing was produced in either case, nor was it obtained in several repetitions of the experiment.

To determine more exactly the influence of different acids upon the bluing of guaiacum by the oxidase of the coffee-bean, a series of experiments were made in the manner already described. In all cases the results obtained were consistent and showed the inhibiting effect was traceable to the activity of the hydrogen ions from the acids in aqueous solution. We conclude, therefore, that the failure to find oxidases in most plant juices, when the acidity is greater per 10 c.c. than that equal to the alkalinity of 0.6 to 0.8 c.c. of N/10 KOH solution, is due to the effect of the different acids upon the peroxidases, etc., and this influence is probably not specific for the acids, but depends upon their dissociation and consequent yield of hydrogen ions. In the following table we indicate the known comparative accelerating effects of these common acids upon the inversion of sucrose,

and their relative retarding effects upon the oxidase tests. The names are arranged in the order of the corresponding activities:

Acceleration of Sucrose Inversion	Retardation of Oxidase Test
HCl (greatest)	HCl (greatest)
H_2SO_4	H_2SO_4
Citric acid	Citric acid
Acetic acid	Acetic acid.

Summary of General Conclusions

1. The oxidases are of very wide distribution among the flowering plants; peroxidases, especially, being present in about seventy-five per cent. of all the specimens examined, while oxygenases (direct oxidases) are less widely distributed, being found in one-half of the plants used. Catalase may be said to be universally distributed, since there were only a few cases in which it was not found.

2. The leaves, stems, roots and food-storage organs of the plants seemed to contain the greatest amounts of the oxidases. The flowers and fruit were in many cases comparatively poor in oxidases. In regard to the fruits this statement must be qualified because dry seeds of somewhat uncertain age were the only available material of certain species.

3. Our experience with a great many parallel tests, using the different oxidase reagents upon a great variety of vegetable tissues show that all of the reagents seem to detect the same substance or substances, for if one reagent gave a positive test the others generally acted in like manner. The phenolphthalin and indo-phenol reagents gave positive results in more cases than the others. This is undoubtedly due to their greater ease of oxidation, for they are spontaneously oxidized by the air.

4. It is probable that in the presence of acid juices in the plant the latter does not form oxidases or else that they are immediately destroyed by the acid. It was shown that the inhibiting effect of acids upon the action of oxidases seemed to be a function of the concentration of the hydrogen ions.

5. Among plants the chromogens are found to the greatest extent in certain orders such as the Liliales, Orchidales, Ranales, and most frequently of all in the latex plants of the Convol-

vulaceae, Boraginaceae, Labiatae, Solanaceae, Rubiaceae, Compositae, etc. Active oxidases are also likely to be associated with chromogens in the latex plants. These conclusions are interesting because of the bearing they have upon Palladin's theory that these chromogens play an important part in the respiration and the metabolism of plants.

The writer wishes to express his deep indebtedness to Professor William J. Gies for suggesting the nature of this investigation and for the aid received from him during its course. The sincere thanks of the writer are likewise due to Doctor N. L. Britton of the New York Botanical Garden, for material obtained from the Conservatories, and also for the other privileges of the institution.

Supplementary Bibliography of Papers Recently Published

Bailey. Oxidizing enzymes and their relation to "sap-stain" in lumber. Bot. Gaz. **50**: 142. 1910.

Bassett and Thompson. The preparation and properties of an oxidase occurring in fruits. Jour. Am. Chem. Soc. **33**: 416. 1911.

Battelli and Stern. Recherche sur la fonction de la catalase. Compt. Rend. Soc. Biol. **68**: 811. 1910.

Bertrand and Rosenblatt. Sur la temperature mortelle des tyrosinases végétales. Compt. Rend. Acad. Sci. **150**: 1142. 1910.

Betting. Oxidase and peroxidase in tobacco. Reviewed in Chem. Abstracts **5**: 740. 1911.

Combes. Du role de l'oxygene dans la formation et la destruction des pigments rouges anthocyaniques chez les végétaux. Compt. Rend. Acad. Sci. **150**: 1186. 1910.

Dox. Catalase of molds. Jour. Am. Chem. Soc. **32**: 1357. 1910.

Gramenisky. Ueber die Widerstandfähigkeit der Oxydasen. Reviewed in Zentralblt. f. Biochem. u. Biophysik. **1**: 606. 1910.

Harter. Starch content of leaves dropped in autumn. Plant World **13**: 144. 1910.

Hoffman and **Sokolowski.** Vergleichenden Atmungsversuche mit verschiedenen Kartoffelsorten. Zeitsch. f. Spiritusindustrie **33**: 391. 1910.

Huber. Ueber die Lebensdauer der Oxydationsenzyme in der Birnenfrüchte. Schweiz. Wochensch. f. Chem. u. Pharm. **48**: 393. 1910.

Kostytschew. Ein eigentümlicher Typus der Pflanzenatmung. Zeitsch. f. Physiol. Chem. **65**: 350. 1910. See also Zeitsch. f. Physiol. Chem. **67**: 116. 1910.

Nalli. Sulla sede intracellulare del fermento ossidante. Clinico Med. Ital. **48**: 24. 1909.

Palladin. Synergin das Prochromogen der Atmungspigmente der Weizenkeime. Biochem. Zeitsch. **27**: 442. 1910.

——. Ueber die Wirkung von Giften auf die Atmung lebender und abgetöteten Pflanzen, sowie auf Atmungsenzmye. Jahrbuch f. Wiss. Bot. **49**: 431. 1910.

—— and **Stanewitsch.** Die Abhängigkeit der Pflanzenatmung von Lipoiden. Biochem. Zeitsch. **26**: 351. 1910.

Rosenberg. Ueber die Rolle der Katalase in den Pflanzen. Ber. Bot. Gesell. **28**: 280. 1910.

Schreiner. Reduction by Roots. Bot. Gaz. **51**: 121. 1911.

—— and **Sullivan.** Studies in soil oxidation. Bull. 73, Bureau of Soils, U. S. Dept. of Agriculture. Washington, 1911.

Sée. Les diastases oxydants et réductrices des champignons. 1–39. Paris, 1910.

Sjollema. Ueber die Bedeutung kolloider Manganoxydlösungen bei biochemischen Oxydationen. Reviewed in Chem. Zentralblt. 1911, I, p. 496.

Wolff. Action des phosphates alcalins bibasiques sur la tyrosinase. Compt. Rend. Acad. Sci. **150**: 477. 1910.

——. Contribution à la connaissance de divers phénomènes oxydasiques naturels et artificiels, 1–99. Paris, 1910.

Zaleski. Ueber die Rolle der Reduktionsprozesse bei der Atmung der Pflanzen. Ber. Bot. Gesell. **28**: 319. 1910.

—— and **Reinard.** Zur Frage der Wirkung der Salze auf die Atmungsenzyme. Biochem. Zeitsch. **27**: 450. 1910.

Laboratory of Biological Chemistry of Columbia University, College of Physicians and Surgeons, New York.

SOME FLORAL FEATURES OF MEXICO*

By H. H. Rusby

(*Continued from April Torreya*)

One of the most beautiful spots that I have ever visited is that of the lava beds a few miles south of Mexico City, on the railroad leading to Cuernavaca. This has been one of the favor-